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Metabolism of Ecdysteroids by a Chitin-Synthesizing Insect Cell Line

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A chitin-synthesizing cockroach cell line (UMBGE-4) previously shown to secrete ecdysteroids was analyzed for its ability to metabolize potential precursors of ecdysone (e.g., 2-deoxyecdysone, 2,22-dideoxyecdysone, 2,22,25-trideoxyecdysone, and cholesterol). All, except cholesterol, were actively metabolized by UMBGE-4 cells. However, all but 2-deoxyecdysone were converted to polar and hydrolyzable metabolites, and not to ecdysone. Labeling with cholesterol was unsuccessful. Labeling experiments with molting hormones, i.e., ecdysone and 20-hydroxyecdysone, confirmed that this cell line can metabolize ecdysteroids and allowed identification of some of the products. Molting hormones were converted into acetate conjugates and polar conjugates which were often double-conjugates, i.e., polar conjugates of acetate conjugates. Labeling experiments with ecdysone demonstrated that this cell line possesses a low ecdysone 20-hydroxylase activity. The capacity of UMBGE-2 cells, which do not synthesize chitin or ecdysteroids, was also examined. Neither ecdysone nor 20-hydroxyecdysone was significantly metabolized by UMBGE-2 cells. 2-Deoxyecdysone and 2,22-dideoxyecdysone were very slowly metabolized respectively to more polar compounds.

Key words: *Blatella germanica*, ecdysone, cholesterol, tissue culture, cell culture

INTRODUCTION

The initiation of chitin synthesis is under the control of ecdysone and its metabolite, 20-hydroxyecdysone, which is more active in most insects [1]. Most in vitro chitin synthesis requires the addition of exogenous ecdysteroids or

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the priming of the tissue with hormones in vivo before explantation. The chitin-synthesizing cell line, UMBGE-4, derived from *Blatella germanica* embryos [2], initially did not seem to require ecdysteroids; however, it was later shown that the cells actually produce an ecdysteroid [3].

Initial analyses of the ecdysteroid products of this cell line showed that the ecdysteroids are secreted into the medium and not retained within the cells as occurs with another ecdysteroid-synthesizing cell line, IAL-TND1, isolated from a lepidopteran [4]. Analysis of the medium from 11-day-old UMBGE-4 cultures showed that ecdysone and small amounts of 20-hydroxyecdysone were present [3].

In order to validate these data and in the hope of using this cell line as a cellular paradigm to determine pathways of ecdysone synthesis, labeling experiments with putative ecdysone precursors (precursors such as 2dE,* or 2,22dE, or 2,22,25dE, and cholesterol) were performed.

MATERIALS AND METHODS

Chemicals

E and 20E were from Simes (Milan, Italy). Acetate reference compounds were obtained by chemical synthesis [5]. [^{3}H] E and [^{3}H] 20-E were prepared by incubating [22,23,24-^{3}H] 2-dE with *Locusta migratoria* Malpighian tubules [6]. [22,23,24-^{3}H] 2-DE, [22,23,24-^{3}H] 2,22-dE, and [22,23,24,25-^{3}H] 2,22,25-dE (specific activity ca. 100 Ci/mmol) were gifts from Dr. C. Hetru (Strasbourg, France). [1α,2α-^{3}H]Cholesterol (specific activity 50 Ci/mmol) was from CEA (Saclay, France).

Cell-Culture

UMBGE-4 and UMBGE-2 cells as described by Kurtti [7] were grown in the cockroach UMN-B1 medium (Hazelton Products, Lenexa, KS) and supplemented with 10% FBS (Grand Island Biological Co., Grand Island, NY). The line 4 cells had been passaged at least 325 times while the line 2 cells had at least 455 subcultures. All experiments were conducted at 26 ± 1°C.

Labeling and Ecdysteroid Extraction

Radiolabeled ecdysteroids were dissolved in 20 μl of 70% ethanol and were added to cultures containing 5 ml of medium. Medium was collected at appropriate times and ecdysteroids extracted. Five micrograms of either E, 20E, or 2dE was added as internal standards to medium samples before extraction. The ecdysteroid extracts were prepared by adsorbing the ecdysteroids onto C_{18} Sep-Pak cartridges (Waters Associates, Milford, MA) and eluting with methanol [8]. Methanol was removed by evaporation and the ecdysteroids analyzed by chromatography.

*Abbreviations used: 2dE = 2-deoxyecdysone; 2,22dE = 2,22-dideoxyecdysone; E = ecdysone; FBS = fetal bovine serum; 20E = 20-hydroxyecdysone; 26E = 26-hydroxyecdysone; 2,22,25-dE = 2,22,25-trideoxyecdysone (5β-ketodiol); NP HPLC = normal phase HPLC; RP HPLC = reverse phase HPLC; RRT = relative retention time; TFA = trifluoroacetic acid.

Incubation of UMBGE-4 cells with labeled cholesterol was accomplished in two ways. The first method involved adding 100 μCi [^{3}H]-cholesterol to line 4 cells in 5 ml of serum-free medium with 0.001% Tween 80 (previously determined to be nontoxic to the cells) to solubilize the cholesterol. The second method involved aseptically incubating 200 μCi[^{3}H]cholesterol with 2.5 ml media containing 20% FBS at room temperature for 16 h. The solution was briefly sonicated to facilitate solubilizing the cholesterol. Medium was decanted without disturbing any of the sediment. Subsequent measurements of the radioactivity indicated that 14% of the cholesterol had been solubilized. UMBGE-4 cells were incubated for 8 days with these cholesterol preparations and the ecdysteroids examined for any incorporation of label.

Chromatographic Analyses

TLC analyses were performed on Kieselgel $6OF_{254}$, 0.25 mm (Merck, Darmstadt, FGR) with chloroform/methanol (80:20). The plates were analyzed using a Berthold radioactivity scanner (model LB 2722; Wildbad, FGR). Ecdysteroids were scraped from the plates and eluted with methanol.

HPLC was performed using either a Kratos instrument (Kratos, Ramsey, NJ) comprised of a Spectroflow 400 pump, a Spectroflow 430 gradient former, and a Spectroflow 757 absorbance detector, or with a Waters instrument, equipped with two 6000A pumps, a M720 gradient former, and a M440 absorbance detector. Radioactivity in the effluent was analyzed either in-line by a Flo-one model IC radioactivity monitor (Radiomatic, Tampa, FL), or by fraction collection with a LKB Redirac collector (LKB, Bromma, Sweden) and subsequent counting. Ecdysteroids were separated using either: 1) RP HPLC on a Novapak C_{18} radial compression column (Waters) with a gradient of acetonitrile either in TFA 0.1% or Tris/$HClO_4$ buffer (20 mM, pH 7.5); or 2) NP HPLC (Zorbax-SIL column, 25 cm × 4.6 mm, particle size 5 μm; DuPont de Nemours, Wilmington, DE) eluted at 1 ml·min^{1} with dichloromethane/propanol-2/water either at 125:25:2 or 125:40:3.

Enzyme Hydrolysis of Conjugates

Putative conjugates were incubated overnight at 30°C in 2 ml of 50 mM potassium acetate buffer pH 4.5 to which 20 μl of *Helix pomatia* juice (Merck) were added. After hydrolysis, ecdysteroids were extracted and purified as described above.

RESULTS

E Precursor Metabolism by UMBGE-4 Cell Line

Line 4 cells metabolized radiolabeled 2dE to several products, one of which comigrated with E (Fig. 1). 2dE was slowly metabolized (Fig. 1, inset). The increase in E did not exactly correspond with the decrease in 2dE. 2,22dE and 2,22,25dE were efficiently metabolized (Fig. 2), but no E was produced. Metabolites from 2dE and 2,22dE were also analyzed by TLC (Fig. 3). The plates were scanned and zones separated as indicated. The extract of each zone was analyzed by RP HPLC in an acetonitrile gradient containing either Tris/$HClO_4$ or TFA. Polar zones derived from 2dE and 2,22dE incubations contained many

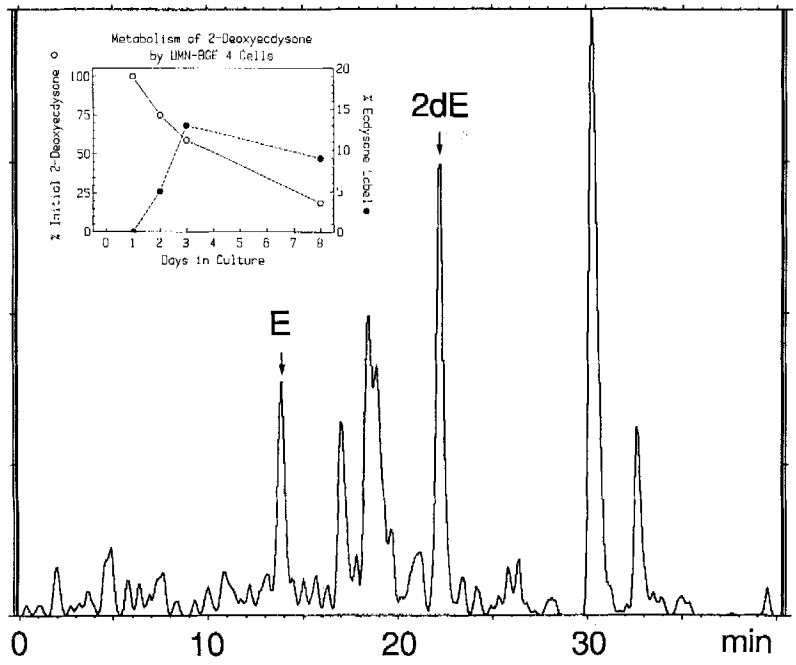

Fig. 1. RP HPLC analysis of 2dE metabolites produced by UMBGE-4 cells after an 8-day incubation. Operating conditions: Novapak C_{18} radial compression column; elution gradient of acetonitrile (18–40% in 30 min then 40–100% in 30 min) in TFA 0.1%; UV 254 nm and [^{3}H] monitoring by Flo-one on-line detector. Inset: Kinetics of the metabolism of 2dE by UMBGE-4 cells.

compounds (Fig. 4), which represented polar conjugates of 2dE and 2,22dE. This conclusion was made since the compounds were ionizable (their retention on RP HPLC changes with the pH of the mobile phase - Fig. 4), and they could be hydrolyzed to 2dE and 2,22 dE upon treatment with *H. pomatia* juice (data not shown).

Incubation with labeled cholesterol did not result in labeling of ecdysteroids.

E and 20E Metabolism by UMBGE-4 Cells

Although only radiolabeled 2dE was converted to E, the precursor experiments demonstrated that line 4 cells metabolized three of these compounds efficiently. We then examined whether these cells also metabolize molting hormones. Indeed, the metabolism of radiolabeled E and 20E proceeded at constant and similar rates over a 2-week incubation experiment (Fig. 5).

The metabolism of E and 20E by line 4 cells was as complex as that of their precursors. Metabolites were subjected to TLC (Fig. 6A, B) and, after radioscanning, were divided into apolar, polar, and molting hormone zones. Figure 7A–C represents the HPLC radioactive profiles of the three zones obtained with E. Each peak was collected and analyzed individually as follows. Compounds from the polar zone were rechromatographed on RP HPLC using Tris/$HClO_4$ buffer and subsequently hydrolyzed by *H. pomatia* juice. Compounds in the apolar and E zones were further analyzed by NP HPLC. Table 1

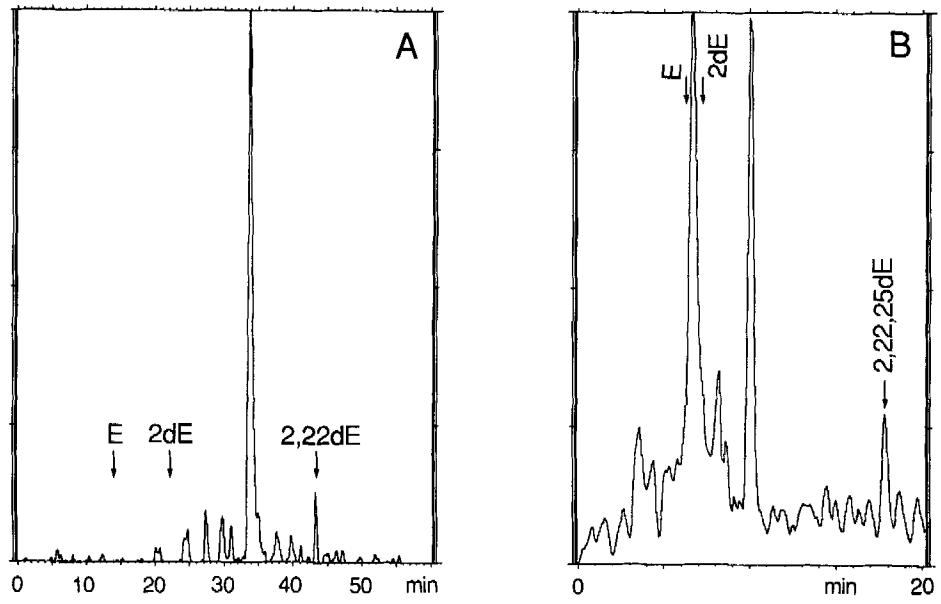

Fig. 2. RP HPLC analysis of 2,22dE (**A**) and 2,22,25dE (**B**) metabolites produced by UMBGE-4 cells after 24-h incubation. Operating conditions: for 2,22dE as Figure 1; for 2,22,25dE: Novapak C_{18} radial compression column; linear elution gradient of acetonitrile/isopropanol (5:2) (30–100% in 30 min) in TFA 0.1%; [^{3}H] monitoring by Flo-one on-line detector.

summarizes the identified metabolites in each zone. The apolar zone contained significant amounts of the E-2-acetate and E-3-acetate. E, 20E, 26E and E-22-acetate were identified in the E zone of the chromatogram. A large number of conjugates were partially identified from the polar zone, including several double conjugates, i.e., polar conjugates of E acetates.

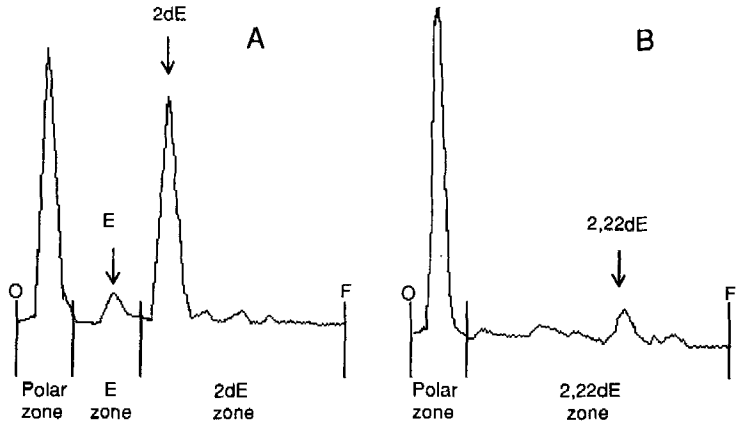

Fig. 3. TLC radio scanner analysis of 2dE (**A**) and 2,22dE (**B**) metabolites produced by UMBGE-4 cells after 24-h incubation. Each zone was scraped and separately analyzed in RP HPLC. Arrows indicate where the reference compounds migrated to. O = origin; F = solvent front.

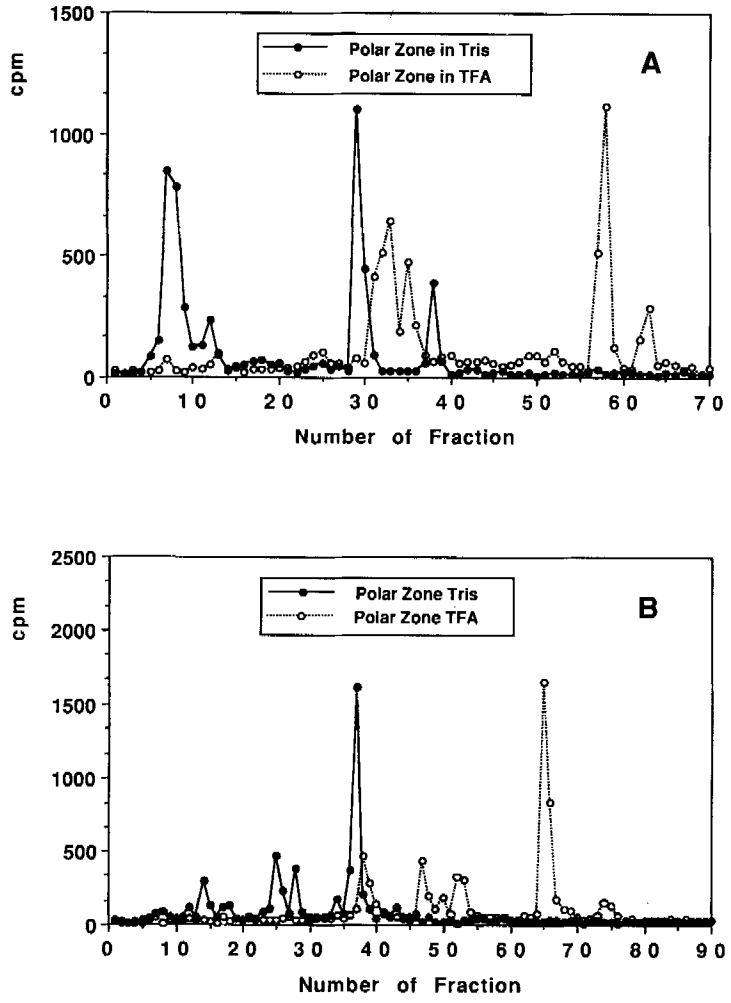

Fig. 4. RP HPLC analysis of polar TLC zones obtained after incubation with either 2dE (**A**) or 2,22dE (**B**) (see Fig. 3). Operating conditions: Novapak C_{18} radial compression column; elution gradient of acetonitrile (18–40% in 30 min then 40–100% in 30 min) in either TFA 0.1% (o) or Tris/$HClO_4$20mM, pH 7.5 (•) 0.5 min fractions were collected.

Table 2 indicates the identified 20E metabolites from each TLC zone (Fig. 6B). The identification criteria for each compound are also presented in Table 2. 20E-3-acetate, 20E-22-acetate, and 20E-2-acetate were identified from the apolar TLC zone. Only 20E was found in the 20E zone, while conjugates of 20E, 20E-3-acetate, and 20E-22-acetate were identified in the polar zone.

Ecdysteroid Metabolism by UMBGE-2-Cells

UMBGE-2 cells, which do not synthesize chitin [9] or ecdysteroids, were tested for their ability to metabolize E and 20E. Even after 8 days in culture, neither E nor 20E was significantly metabolized. There was, however, some

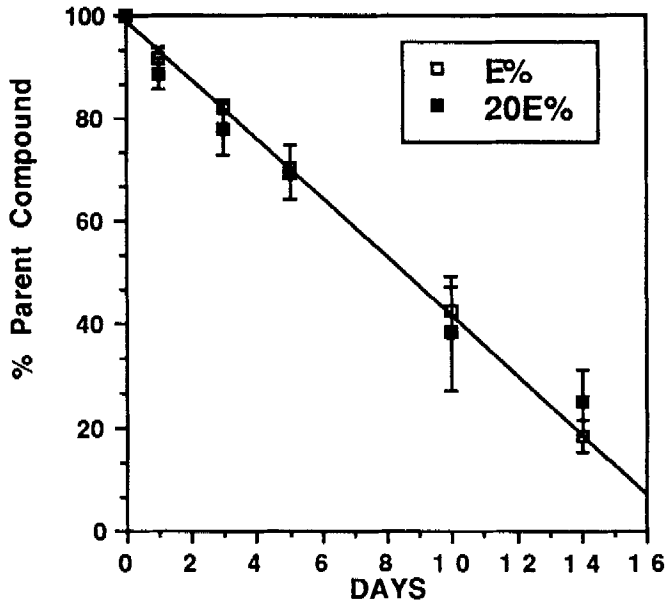

Fig. 5. Metabolism of radiolabeled E and 20E by UMBGE-4 cells over a 2-week period expressed as the % parent compound after various incubation times (± SEM; three replicates).

metabolism of 2dE (11%) into polar compounds after 8 days in culture. When 2,22dE was incubated with line 2 cells, after 1 day, 72% of this compound was converted into a number of more polar metabolites, none of which were 2dE, E, or 20E.

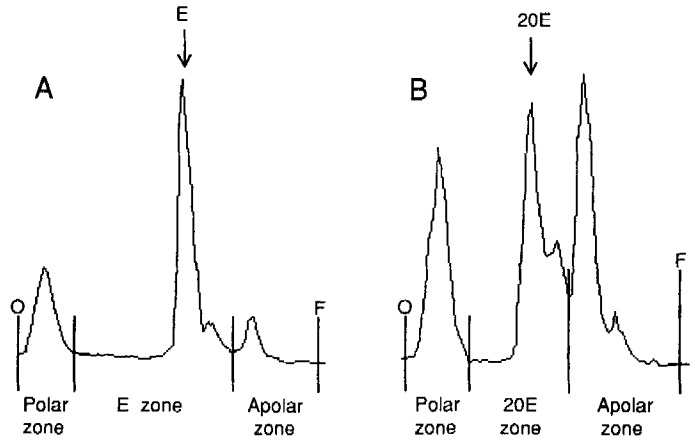

Fig. 6. TLC radio scanner analyses of E (A) and 20E (B) metabolites produced by UMBGE-4 cells after 8 days of incubation. Each plate was divided into three zones, which were further analyzed by HPLC. Arrows indicate where the reference compounds migrated to. O = origin; F = solvent front.

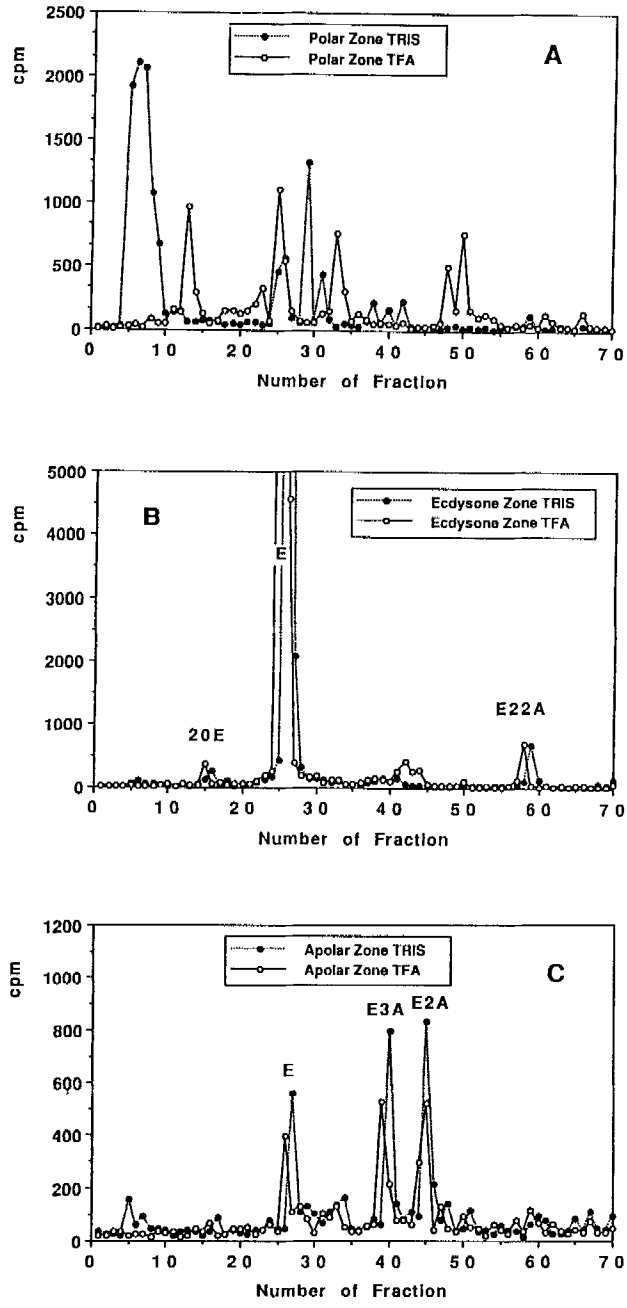

Fig. 7. RP HPLC analysis of the E, polar, and apolar zones obtained after TLC analysis of E metabolites after incubation with UMBGE-4 cells (see Fig. 6A). Operating conditions: Novapak C_{18} radial compression column; elution gradient of acetonitrile (18–40% in 30 min then 40–100% in 30 min) either in TFA 0.1% (o) or Tris/$HClO_4$20mM, pH 7.5 (•) 0.5 min fractions were collected. **A:** polar zone; **B:** E zone; **C:** apolar zone.

TABLE 1. Identification of UMBGE-4 Cell Line E Metabolites Occurring in the Apolar, E and Polar TLC Zones

Zone	RRT TFA[a]	RRT Tris[b]	RRT SIL[c]	Ecdysteroid	Identification criterion
Apolar	1.54	1.54	0.38	E-3-acetate	Comigration with reference
	1.69	1.69	0.39	E-2-acetate	Comigration with reference
E	0.58	0.58	1.34[d]	20E	Comigration with reference
	0.58	0.58	2.13[d]	26E	Comigration with reference
	1	1	1	E	Comigration with reference
	2.30	2.30	0.66	E-22-acetate	Comigration with reference
Polar	0.38	0.23	—	E conjugate	*H. pomatia* hydrolysis
	0.81	0.31	—	E conjugate	*H. pomatia* hydrolysis
	0.88	0.27	—	E3A conjugate	*H. pomatia* hydrolysis
	1.23	0.35	—	Undetermined conjugate	*H. pomatia* hydrolysis
	1.81	1.08	—	E-22-acetate conjugate	*H. pomatia* hydrolysis
	1.92	1.15	—	E-22-acetate conjugate	*H. pomatia* hydrolysis
	2.5	1.5		E-22-acetate conjugate	*H. pomatia* hydrolysis

[a]Relative retention times (RRT) from RP HPLC using 0.1% TFA.
[b]RRT from RP HPLC in Tris/$HClO_4$ buffer.
[c]RRT from NP HPLC using dichloromethane/isopropanol/water (125:25:2).
[d]RRT isooctane from NP HPLC using isooctane/isopropanol/water (125:40:3).

DISCUSSION

We started these experiments to determine the ability of UMBGE-4 cells to synthesize ecdysteroids de novo [3] and to use this cockroach vesicle cell line as an in vitro model to trace the path of E synthesis from cholesterol to putative late precursors such as 2dE. Unfortunately, we were unable to demonstrate full steroidogenic activity in this cell line. Our results with labeled cholesterol were not totally unexpected, since this compound is generally poorly incorporated into in vitro systems. Only a few articles report the successful incorporation of labeled cholesterol in vitro by an ecdysteroid-producing organ, the prothoracic glands of *Manduca sexta* [10] and *Bombyx mori* [11] and *Tenebrio molitor* [12]. In the present case, the respective pool sizes of ecdysteroids (as measured by RIA) and of cholesterol (from FBS) provide much less favorable conditions. In fact, there is no evidence that cholesterol is taken up by the cells.

TABLE 2. Identification of UMBGE-4 Cell Line 20E Metabolites Occurring in the Apolar, 20E, and Polar TLC Zones

Zone	RRT TFA[a]	RRT SIL[b]	Ecdysteroid	Identification criterion
Apolar	1.84	0.40	20E-3-acetate	Comigration with reference
	1.95	0.38	20E-22-acetate	Comigration with reference
	2.16	0.62	20E-2-acetate	Comigration with reference
20E	1	1	20E	Comigration with reference
Polar	0.53	—	20E conjugate	Ionizable and *H. pomatia* hydrolysis
	1	—	20E-3-acetate conjugate	Ionizable and *H. pomatia* hydrolysis
	1.58	—	20E-22-acetate conjugate	Ionizable and *H. pomatia* hydrolysis

[a]RRT from RP HPLC using 0.1% TFA.
[b]RRT from NP HPLC using dichloromethane/isopropanol/water (125:25:2).

The experiments with putative late E precursors were disappointing, since these types of experiments generally work in vivo [13–15] and in vitro when steroidogenic organs are tested [16]. We were only able to demonstrate the presence of the C-2 hydroxylase activity in the UMBGE-4 cells. The metabolism of the other putative precursors of E (2,22dE and 2,22,25dE) was very efficient but E was not one of the products obtained. These experiments indicate that the UMBGE-4 cells are able to metabolize ecdysteroids into inactive conjugates.

Labeling experiments with E and 20E allowed us to determine the nature of ecdysteroid metabolism. The conversion of radiolabeled E to 20E indicates that E 20-hydroxylase is present. The conversion of E to 26E indicates that E 26-hydroxylase is also present. The conjugation processes are very efficient: acetylation occurs at any position, e.g., at C-2, C-3, and C-22. Polar conjugates, the chemical nature of which is still unknown, are also produced.

The fact that putative late E precursors were not converted to E does not signify the absence of steroidogenic ability of line 4 cells. Several explanations can be proposed for this lack of success. Two explanations follow.

1. It is possible that E synthesis takes place only in a few percent of cells which are in the correct physiological state, whereas the other cells not only do not synthesize ecdysteroids but, additionally, inactivate them by conjugation. To test such a hypothesis would require synchronization of these cells. Available data from epidermal cultures from *T. molitor* would be consistent with such a concept [17].
2. An alternative explanation would be the inadequacy of those cells to use precursors used by other insect species. It is indeed conceivable that the order of hydroxylations differs among insect species, due either to different substrate requirements of the monooxygenases or to the inability of some compounds to enter the proper cellular compartment.

There are many examples of responses of insect cell lines to added E or 20E including morphological changes [18], induction of enzymes [19], changes in cell-surface glycoproteins [20], increases in chitin-like or chitin-protein molecules [21], and changes in membrane transport [22]. The best known example of a hormone response is that of the *Drosophila* Kc cell line which responds to 20E by synthesis of specific proteins [23], changes in cell shape [18], induction of β-galactosidase [24], DOPA-decarboxylase [25] and acetylcholinesterase [19], and G2-block of the cell cycle [26] (for a general review, see [27]). The metabolism of ecdysteroids by responding cell lines may mediate or modify the response to the hormone. Indeed, our knowledge of ecdysteroid metabolism by insect cell lines is limited [27]. Kc cells do not metabolize the molting hormones (E or 20E) [27] as is also the case with the UMBGE-2 cell line. However, recently it was demonstrated that a *Plodia* cell line metabolizes molting hormones [28]. In the *Plodia* cell line, E metabolism differs from that of the UMBGE-4 cell line. 20-Hydroxylation is efficient and ecdysonoic acids are formed, but conjugation mechanisms are limited. Of course, these differences reflect differences in E metabolic pathways in the insects, themselves.

It is of interest to determine why these cultured cells metabolize the hor-

mones. The UMBGE-4 vesicle cell culture cells were derived from the embryonic germband stage. It was possible to initiate similar vesicle cultures from 5- to 8-day-old embryos. Attempts to initiate cultures before and after this time period produced no vesicles [G.B. Ward, unpublished data]. Transmission electron microscopy studies revealed that this time period corresponded to the formation of the first embryonic cuticle. As soon as cuticle synthesis ceased, vesicles could not be initiated [G.B. Ward, unpublished data]. In vivo, the formation of embryonic cuticles is correlated with the appearance of ecdysteroid peaks [29]. Ecdysteroids in developing embryos may arise either from maternal sources [29,30] or possibly from synthesis by embryonic tissues [31–33]. If ecdysteroids are involved in embryonic cuticle formation, the tissues may have a role in synthesis and inactivation of those ecdysteroids during this time. This would account for the extensive metabolism of ecdysteroids observed in vitro with UMBGE-4 cell line. It would be of great interest to examine the levels and forms of ecdysteroids during this stage of development and compare this to the abilities of established cell cultures, cultured embryos, and primary cell cultures to metabolize ecdysteroids.

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